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Reversal of BoNT/A-mediated inhibition of muscle paralysis by 3,4-diaminopyridine and roscovitine in mouse phrenic nerve-hemidiaphragm preparations ☆

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ABSTRACT

Botulinum neurotoxins (BoNTs) comprise a family of neurotoxic proteins synthesized by anaerobic bacteria of the genus *Clostridium*. Each neurotoxin consists of two polypeptide chains: a 100 kDa heavy chain, responsible for binding and internalization into the nerve terminal of cholinergic motoneurons and a 50 kDa light chain that mediates cleavage of specific synaptic proteins in the host nerve terminal. Exposure to BoNT leads to cessation of voltage- and Ca^{2+}-dependent acetylcholine (ACh) release, resulting in flaccid paralysis which may be protracted and potentially fatal.

There are no approved therapies for BoNT intoxication once symptoms appear, and specific inhibitors of the light chain developed to date have not been able to reverse the consequences of BoNT intoxication. An alternative approach for treatment of botulism is to focus on compounds that act by enhancing ACh release. To this end, we examined the action of the K^+ channel blocker 3,4-diaminopyridine (3,4-DAP) in isolated mouse hemidiaphragm muscles intoxicated with 5 pM BoNT/A. 3,4-DAP restored tension within 1–3 min of application, and was effective even in totally paralyzed muscle. The Ca^{2+} channel activator (R)-roscovitine (Ros) potentiated the action of 3,4-DAP, allowing for use of lower concentrations of the K^+ channel blocker. In the absence of 3,4-DAP, Ros was unable to augment tension in BoNT/A-intoxicated muscle. This is the first report demonstrating the efficacy of the combination of 3,4-DAP and Ros for the potential treatment of BoNT/A-mediated muscle paralysis.

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1. Introduction

The seven serotypes of botulinum neurotoxin (BoNT) are the most potent substances in nature, and exposure to as little as 1–3 ng/kg may be sufficient to cause human lethality (Arnon et al., 2001; Lindström and Korkeala, 2006; Rega et al., 2010). The neurotoxins are produced by spore forming anaerobic bacteria, chiefly *Clostridium botulinum*, and by a limited number of other clostridial strains (Simpson, 2004; Sobel, 2005). BoNTs are secreted initially as relatively inactive ~150 kDa protoxins (range 140–167 kDa), surrounded by a complex of neurotoxin-associated proteins that protect BoNT from degradation in the gastrointestinal tract (Kukreja and Singh, 2007; Gu et al., 2012). The protoxin is subsequently cleaved to form the active dichain neurotoxin, consisting of a 100 kDa heavy chain (HC) and a 50 kDa light chain (LC) (DasGupta and Sugiyama, 1972).

The BoNTs have three functional domains: binding, translocation and catalytic. Binding is mediated by the C-terminal region of the heavy chain, which interacts with gangliosides and protein receptors located on cholinergic nerve terminals (Dong et al., 2006; Benson et al., 2011); selective binding of BoNT to these receptors is responsible for the cholinergic selectivity of the BoNTs. The N-terminal region of the HC promotes translocation of the LC into the cytosol (Koriazova and Montal, 2003; Fischer and Montal, 2007). The LC is a Zn^{2+}-containing endopeptidase that cleaves specific sites on SNARE (soluble-*N*-ethylmaleamide sensitive factor attachment protein receptor) proteins, leading to a cessation of evoked transmitter release (Montecucco et al., 2005).

All seven serotypes of BoNT (A–G) inhibit release of acetylcholine (ACh) from cholinergic nerve terminals that innervate skeletal muscle, autonomic ganglia and post-ganglionic parasympathetic organs (Simpson, 2004). In skeletal muscle, inhibition of transmitter release leads to flaccid paralysis, which can progress to generalized

☆ The views expressed are those of the authors and do not reflect the official policy of the U.S. Department of Army, Department of Defense, or U.S. Government. The experimental protocol was approved by the animal Care and Use Committee at the United States Army Medical Research Institute of Chemical Defense, and all procedures were conducted in accordance with the principles stated in the Guide for the Care and Use of Laboratory Animals and the Animal Welfare Act of 1966 (P.L. 89-544), as amended. This research was supported by the Defense Threat Reduction Agency-Joint Science and Technology Office, Medical S & T Division.

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muscle weakness and death when the muscles of respiration become sufficiently compromised.

Early signs of BoNT intoxication (botulism) include visual disturbances, difficulties in swallowing and impairment of speech (Sobel, 2005). At this stage, botulism can be treated by infusion of serotype specific antitoxin. However, when symptoms progress to generalized paralysis, antitoxin is no longer effective, and patients will need treatment in an intensive care facility (Tacket et al., 1984; Hatheway et al., 1984). The more severe cases may also require extensive periods of artificial ventilation, enteral feeding and physical therapy after discharge from intensive care (Shapiro et al., 1998; Robinson and Nahata, 2003; Marcus, 2009).

BoNT has numerous attributes that make it appealing to terrorists, including an unusually high potency, long duration of action and the potential to cause widespread panic with major social disruption (Arnon et al., 2001; Robinson and Nahata, 2003). Accordingly, BoNT has been classified as a Tier 1 select toxin by the U.S. Department of Health and Human Services, the only non-infectious agent to receive this designation.

Of the seven serotypes, BoNT/A has the highest potency and longest duration of action and it therefore represents the greatest bioterrorist threat (Arnon et al., 2001). A number of complementary approaches have been used to develop pharmacological antagonists for BoNT/A: these consist of synthesis and screening of small molecule inhibitors (SMIs) for their ability to inactivate the BoNT/A LC, strategies to enhance degradation of the LC from intoxicated nerve terminals and evaluation of physiological antagonists such as the K^+ channel blocker 3,4-diaminopyridine (3,4-DAP) and the Ca^{2+} channel activator (R)-roscovitine (Ros) that can overcome the inhibitory action of BoNT and restore ACh release. To date, SMIs have only been able to slow the rate of BoNT/A-mediated paralysis, but they have not proved effective in reversing the paralytic action of BoNT, and strategies to accelerate the removal of BoNT/A LC from the nerve terminal have not progressed beyond the proof-of-concept stage (Tsai et al., 2010). However, physiological antagonists have shown remarkable promise both in slowing the onset of muscle paralysis and in restoring tension in BoNT/A-paralyzed muscles (Molgó et al., 1980, 1987; Adler et al., 1995, 1996; Mayorov et al., 2010).

A notable advantage of developing 3,4-DAP and Ros for treatment of botulism is that both have an extensive history of clinical use: aminopyridines for the symptomatic treatment of multiple sclerosis, Lambert Eaton Myasthenic Syndrome (LEMS) and downbeat nystagmus (Shi and Sun, 2011; Kalla et al., 2011), and Ros for breast, ovarian and prostate cancer (Benson et al., 2007; Yarotsky and Elmslie, 2012). In addition, 3,4-DAP and other K^+ channel blockers have been investigated for treatment of human botulism, with some success (Puggiari and Cherington, 1978; Kalia and Swartz, 2011).

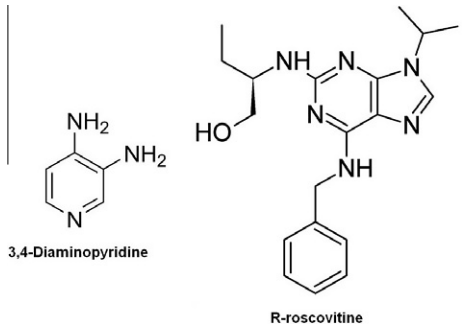

Fig. 1. Structure of the K^+ channel blocker 3,4-DAP and the Ca^{2+} channel activator Ros. 3,4-DAP (CAS No. 54-96-6) is a *N*-heterocyclic tertiary amine also known as amifampridine in clinical use. The phosphate salt was licensed in Europe as an orphan drug for treatment of rare muscle disorders such as LEMS in 2010. Ros (CAS No. 186692-46-6) is a 2,6,9-tri substituted purine analog that is marketed under the proprietary name Seliciclib primarily as an antitumor agent.

The current study was undertaken to examine the ability of 3,4-DAP and Ros (Fig. 1) to reverse muscle paralysis produced by BoNT/A in isolated mouse hemidiaphragm muscle. The results indicate that 3,4-DAP can restore muscle tension completely, and combinations of 3,4-DAP with Ros allow for use of lower, less toxic concentrations of the former. It is concluded that 3,4-DAP and Ros are promising lead compounds for the development of medical countermeasures for botulism. This is the first study examining the efficacy of Ros in BoNT intoxication, as well as the first showing efficacy of co-administering Ros and 3,4-DAP in reversing BoNT/A-mediated muscle paralysis.

2. Methods

2.1. Muscle preparation

Experiments were performed *in vitro* on isolated hemidiaphragm muscles dissected from adult male CD-1 mice (19–24 g on arrival; Charles River Laboratories, Wilmington, MA, USA). Mice were housed in facilities approved by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC) with food and water provided *ad libitum*. Animals were euthanized by decapitation following exposure to excess isoflurane. Hemidiaphragms with attached phrenic nerves were mounted in 20-ml tissue baths containing Tyrode's solution of the following composition (mM): NaCl, 137; KCl, 5; $MgSO_4$, 1; $NaHCO_3$, 24; NaH_2PO_4, 1; $CaCl_2$, 1.8, and glucose, 11. The solution was bubbled with a gas mixture of 95% O_2/5% CO_2 yielding a pH of 7.3–7.4. Resting tension was maintained at 0.7 g to generate optimal nerve-evoked contractions.

2.2. Tension recordings

Twitch tension was elicited by supramaximal stimulation of the phrenic nerve via bipolar, stainless steel electrodes (6.0–9.0 V, 0.2 ms duration) at 0.033 Hz. Tetani were elicited by repetitive stimulation at 30 Hz for 1 s, with 1-min intervals between stimulus trains. Muscle tensions were measured using Grass FT03 force displacement transducers (West Warwick, RI, USA), digitized and analyzed offline using pClamp software v. 10.1 (Molecular Devices, Sunnyvale, CA, USA). Following a 15- to 20-min equilibration, muscles were exposed to 5 pM of pure BoNT/A (Metabiologics, Inc., Madison, WI, USA) for 30 min at room temperature (18–22 °C) in the absence of nerve stimulation. The muscles were then washed with control Tyrode's solution to remove unbound BoNT/A, warmed to 36 °C and monitored for development of paralysis 3,4-DAP (Sigma–Aldrich, St. Louis, MO, USA) and Ros (A.G. Scientific, Inc., San Diego, CA, USA) were generally added to the bath when muscle tensions declined to ~50% of their initial values. Tensions were recorded for 1–3 h after drug addition to determine the time course for reversal of muscle paralysis.

2.3. Drug preparation

Stock solutions of 3,4-DAP were prepared in deionized water at concentrations of 20 mM. Stock solutions of Ros were prepared in dimethylsulfoxide (DMSO) at a concentration of 50–100 mM and stored in opaque vials to limit their photoreactivity. Stock solutions were stored at 4 °C.

2.4. Data analysis

Unless stated otherwise, all data are expressed as means ± SEM. Statistical analysis was performed using a one-way analysis of variance (ANOVA) followed by the Bonferroni multiple comparison

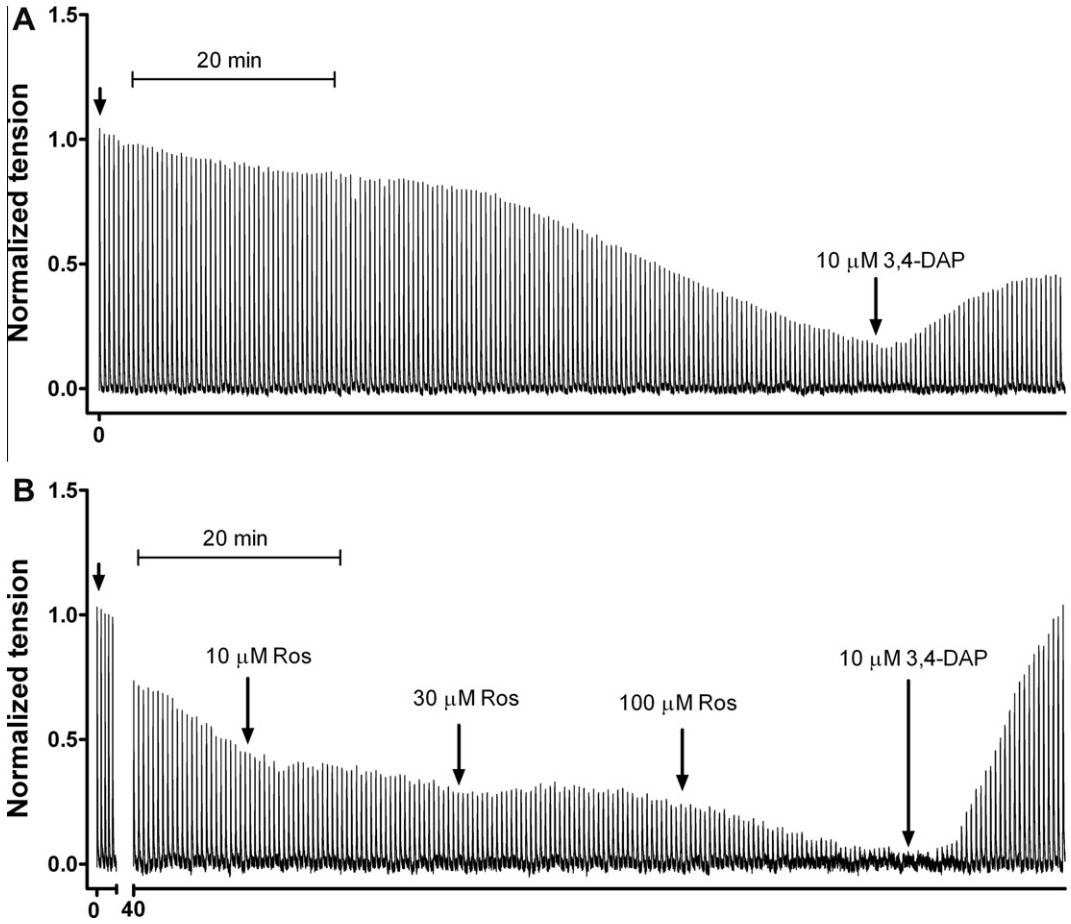

Fig. 2. Recordings of neurally-elicited twitch tensions in mouse hemidiaphragm muscle. (A) The muscle was intoxicated with BoNT/A (5 pM) at 0-time (short arrow), and 3,4-DAP (10 μM) was added when tension had declined to 17% of control. 3,4-DAP (10 μM) produced a partial restoration of tension. Tensions were normalized by averaging 10 control responses just prior to BoNT exposure, and setting these equal to 1. (B) Recordings illustrating the effects of Ros on a hemidiaphragm muscle intoxicated with 5 pM BoNT/A (short arrow). Ros was added in increasing concentrations at the indicated time points. Addition of 10 μM 3,4-DAP in the presence of 100 μM Ros led to complete restoration of tension. Tensions were normalized by averaging 10 control responses just prior to BoNT/A, and setting these equal to 1. Contractions elicited 2.5–40 min after addition of BoNT/A were omitted for clarity.

test (GraphPad, InStat, La Jolla, CA, USA). $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Time course of BoNT/A-mediated paralysis and reversal by 3,4-DAP

Twitch tension in control mouse hemidiaphragm muscle ranged from 0.7 to 2.2 g and was stable with time, decreasing by less than 10% over 4 h. Exposure to 5 pM BoNT/A led to a progressive reduction in tension after a variable latent period, culminating in total muscle paralysis (half-time = 61.7 ± 5.8 min) (Fig. 2). In this hemidiaphragm, 10 μM 3,4-DAP was added 77 min after 5 pM BoNT/A, when twitch tension had declined to less than 20% of control. Tension increased within 2 min of 3,4-DAP addition and continued to rise for the next 15 min before attaining steady-state at 45% of control. The ability of 3,4-DAP to reverse muscle paralysis is of considerable interest since no SMI examined to date has been able to increase tension in BoNT/A-intoxicated muscle after onset of paralysis (Adler and Nicholson, 2008; Li et al., 2010; Ruthel et al., 2011).

3.2. Effect of Ros and Ros–3,4-DAP combination on muscle tension

In the absence of 3,4-DAP, Ros was unable to halt the progression of BoNT/A-mediated depression of twitch tension at any concentration tested (10–100 μM). Fig. 2B illustrates the effect of Ros in a hemidiaphragm muscle intoxicated with 5 pM BoNT/A. Addition of 10 or 30 μM Ros led to a transient increase in tension, followed by resumption of paralysis. Raising the concentration of Ros to 100 μM failed to produce even a transient increase, and twitches continued to decline to a level where they could barely be distinguished from baseline noise (Fig. 2B). A subsequent addition of 10 μM 3,4-DAP to this preparation at a time of near complete muscle paralysis produced a rapid restoration of tension. From comparison of Fig. 2A and B, it is clear that Ros markedly augments the action of 10 μM 3,4-DAP in BoNT/A-intoxicated muscle.

Data illustrating the interaction of 3,4-DAP and Ros in 11–16 hemidiaphragm muscles are shown in Fig. 3. In control muscle, twitch tension was potentiated after addition of either 30 μM Ros or 10 μM 3,4-DAP (Fig. 3A): the former to 128% of control ($P < 0.05$) and the latter to 230% of control ($P < 0.001$). Addition of 30 μM Ros to the Tyrode's solution containing 10 μM 3,4-DAP elevated tensions to 287% of control ($P < 0.05$, combination relative to 3,4-DAP alone).

The individual drugs and combination were subsequently tested in muscles intoxicated with 5 pM BoNT/A (Fig. 3B). The drugs were added after muscle tensions had declined to 54 ± 4% of control and evaluated for their ability to restore tension after 20- to 30-min of incubation. No increase in twitch tension was observed in BoNT/A-intoxicated muscle in the presence of 30 μM Ros (Fig. 3B), consistent with the data in Fig. 2B. In contrast, addition of

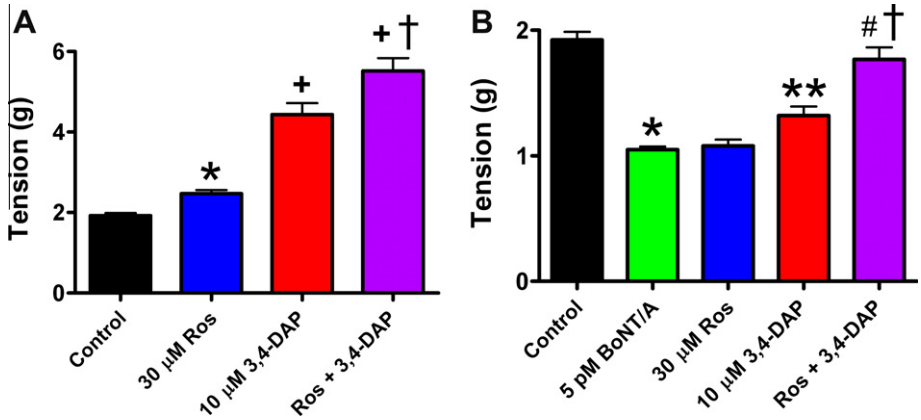

Fig. 3. Histograms showing the effects of 30 μM Ros, 10 μM 3,4-DAP and combination of the two drugs in control hemidiaphragm muscles (A) and in hemidiaphragm muscles intoxicated with 5 pM BoNT/A (B). Data shown are mean ± SEM of supramaximal twitch tensions obtained from 11 to 16 muscles per condition. Recording were made 20–30 min after drug addition. Muscles were washed for 40 min with control Tyrode's solution between Ros and 3,4-DAP addition. (A) Differs significantly from control ($^{*}P < 0.05$, $^{+}P < 0.001$); differs significantly from values observed in 10 μM 3,4-DAP ($^{\dagger}P < 0.05$). (B) Differs significantly from control ($^{*}P < 0.001$); differs significantly from values in the presence of BoNT/A ($^{**}P < 0.05$, $^{\#}P < 0.001$); differs significantly from values observed in 10 μM 3,4-DAP ($^{\dagger}P < 0.001$).

10 μM 3,4-DAP increased muscle tension by 26% ($P < 0.05$). Addition of 30 μM Ros to bathing medium containing 10 μM 3,4-DAP led to an even greater increase in tension ($P < 0.001$, combination vs. 3,4-DAP alone). Co-application of these two drugs was thus able to restore tension to near control levels (Fig. 3B).

3.3. Effects of high concentrations of 3,4-DAP

Since 3,4-DAP can reverse the inhibitory action of BoNT/A on its own, it may be questioned as to whether addition of a second drug is necessary. Although 3,4-DAP is fully effective (Molgó et al, 1980), the high concentrations required generally result in abnormal contractions, as depicted in Fig. 4. The traces were obtained from a hemidiaphragm muscle under control conditions and after sequential additions of BoNT/A and 3,4-DAP at the indicated concentrations. Exposure of the muscle to 5 pM BoNT/A led to a profound reduction in twitch tension (Fig. 4B) as noted earlier (Fig. 2). Addition of 30 μM 3,4-DAP caused a marked enhancement of tension with no change in the time course (Fig. 4C). Raising the 3,4-DAP concentration to 100 μM led to a further increase in twitch tension, accompanied by abnormal repetitive after-discharges (Fig. 4D). Such after-discharges act to prolong the relaxation phase of twitches, thus interfering with the ability of muscles to follow higher stimulation frequencies. By combining 3,4-DAP with Ros, it was possible to reverse the paralytic effect of BoNT/A without altering the normal characteristics of muscle twitches (Fig. 2B).

3.4. Effect of 3,4-DAP and Ros on tensions elicited by repetitive stimulation

Since tension in skeletal muscle is normally generated by repetitive firing of motoneurons, it was of interest to evaluate the actions of 3,4-DAP, Ros and the combination of the two drugs under conditions of repetitive stimulation. Data obtained with 30 Hz trains are shown in Fig. 5. This frequency was selected because it is within the physiological range for mouse phrenic motoneurons (Paton, 1997). In response to 30 Hz stimulation, control muscles exhibited a sustained plateau, with superimposed cycles of contraction and relaxation (Fig. 5A). Exposure to 5 pM BoNT/A led to a reduction in tetanic tension amplitude and area (Fig. 5B). Addition of 30 μM Ros to the BoNT/A-intoxicated muscle resulted in a small increase in tetanic tension (Fig. 5C), and a more pronounced increase was observed after addition of 10 μM 3,4-DAP (Fig. 5D) As with twitch tensions, 30 μM Ros potentiated the action of 10 μM 3,4-DAP (Fig. 5E). A high concentration of 3,4-DAP (100 μM) elicited an even more dramatic enhancement of tetanic tension; however, both the plateau and baseline following the train exhibited instability (Fig. 5F).

Data from 5 to 9 such experiments are shown in Fig. 6. The ability of Ros and 3,4-DAP to reverse the action of 5 pM BoNT was assessed after tetanic area was reduced to 51 ± 3% of control. Addition of 30 μM Ros increased tetanic area by a small but not significant amount (19%), whereas 10 μM 3,4-DAP augmented tetanic area by 61% ($P < 0.05$). Addition of Ros to the 3,4-DAP-containing solution led to a further increase, such that in the presence of both drugs, tetanic area was 122 ± 12% of control ($P < 0.01$, combination vs. 3,4-DAP).

4. Discussion

The results of this investigation demonstrate that K^+ channel blockers and Ca^{2+} channel activators have important roles as potential medical countermeasures for the treatment of botulism. Of the two classes of drugs, the K^+ channel blockers have been studied more extensively. Their antagonism of BoNT/A-mediated paralysis stems from their ability to prolong the duration of the nerve terminal action potential (Penner and Dreyer, 1986), leading to a greater influx of Ca^{2+} during nerve stimulation. Coupling of increased Ca^{2+} influx to nerve impulses enables the K^+ channel blockers to produce striking increases in the amplitude of endplate potentials (EPPs) and of nerve-evoked twitch tensions (Lundh et al., 1977; Adler et al., 1979, 1995).

4.1. Role of K^+ channel blockers in antagonizing BoNT/A-mediated muscle paralysis

A number of K^+ channel blockers have been evaluated for their ability to antagonize the actions of BoNT, including guanidine, 4-aminopyridine, 3,4-DAP and tetraethylammonium (Cherington and Ryan, 1968; Cherington and Schultz, 1977; Lundh et al., 1977; Molgó et al., 1980, 1987; Simpson, 1986). Aminopyridines and tetraethylammonium inhibit different K^+ channel subtypes at the mammalian motor nerve terminal (Penner and Dreyer, 1986; Lin and Lin-Shiau, 1997; Nakamura and Takahashi, 2007), and both would appear to be useful for counteracting the inhibitory action of BoNT on transmitter release. Of the K^+ channel blockers examined, the most promising candidate thus far has been 3,4-DAP. 4-Aminopyridine produced a higher incidence of undesirable central

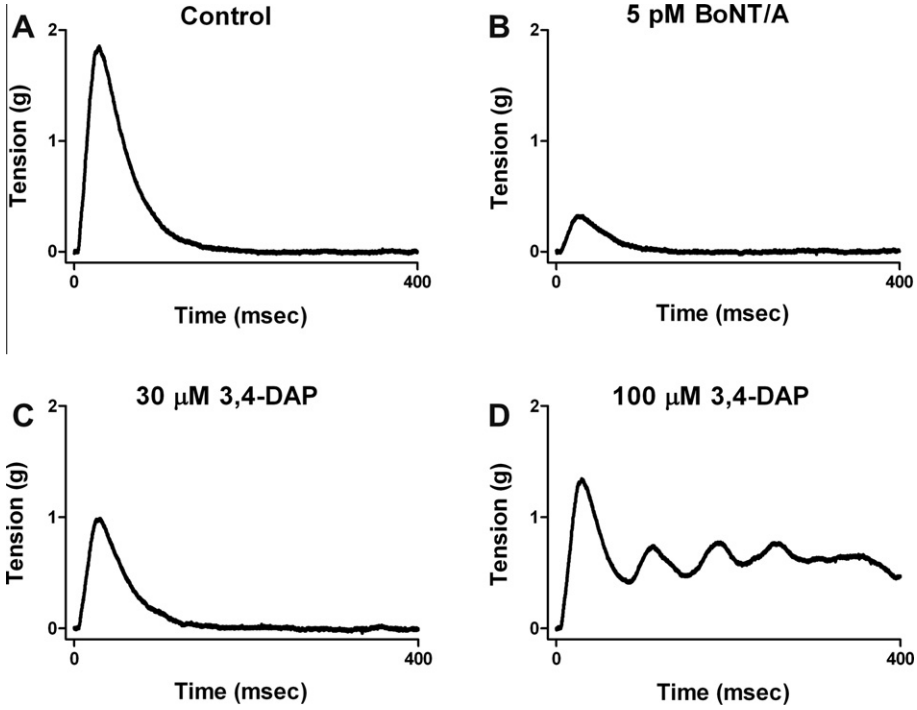

Fig. 4. Traces showing the effect of increasing concentrations of 3,4-DAP on the amplitude and time course of single twitches. The records were obtained from a hemidiaphragm muscle intoxicated with 5 pM BoNT/A and incubated sequentially with 30 and 100 μM 3,4-DAP. The time course of twitches were unaltered by BoNT or 30 μM 3,4-DAP, whereas 100 μM 3,4-DAP produced a series of after-discharges that prolonged the relaxation phase.

nervous system (CNS) side effects, presumably because of its greater lipid solubility (Molgó et al., 1980). Tetraethylammonium, although devoid of CNS toxicity, has been found to cause a marked inhibition of muscle ACh receptor channels, thereby exacerbating BoNT/A-mediated muscle paralysis (Adler et al., 1979, 1995; Thesleff, 1989). When added to nerve-muscle preparations prior to BoNT, 3,4-DAP produced a marked delay in the time-to-block of nerve-evoked muscle contractions (Simpson, 1986; Mayorov et al., 2010). When applied after BoNT, 3,4-DAP was able to elevate tensions to control values or above (Figs. 2 and 3). In addition, unlike other putative BoNT/A antagonists, 3,4-DAP could restore tension even in muscles that were paralyzed for 2 weeks prior to drug administration (Adler et al., 1996).

4.2. Limitations of K^+ channel blockers in BoNT intoxication

In spite of these successes, 3,4-DAP also has limitations: (1) the efficacy of 3,4-DAP is largely confined to serotype A (Simpson, 1986; Adler et al., 1996), (2) concentrations of 3,4-DAP that are effective in reversing BoNT/A intoxication are toxic to humans (Bever et al., 1990; Vollmer et al., 2009), and (3) 3,4-DAP has a brief *in vivo* half-life relative to that of BoNT/A (Adler et al., 1996). The short duration of action can be offset, however, by use of an infusion delivery as demonstrated by Adler et al. (2000) with subcutaneously implanted osmotic minipumps. In addition, slow release formulations of the aminopyridines are currently available (Korenke et al., 2008; Vollmer et al., 2009; Hayes, 2011).

The basis for the lack of response to 3,4-DAP by the other serotypes is not well understood. At a functional level, BoNT/A-intoxicated neuromuscular junctions undergo an attenuated but synchronous release of ACh following stimulation; preparations intoxicated by serotypes B, D and F produce asynchronous release where the ACh quanta are dispersed and cannot summate to produce suprathreshold EPPs (Lundh et al., 1977; Molgó et al., 1980; Thesleff, 1989).

It is readily apparent that the lack of synchrony would prevent 3,4-DAP from restoring transmitter release; however, the factors that lead to asynchronous release are still not well understood. In this context, it is of interest that homozygous SNAP-25 null neurons were able to generate only asynchronous EPSCs; fast synchronous EPSCs could be restored, however, by lentiviral transfection with SNAP-25 (Delgado-Martínez et al., 2007). Collectively, these observations suggest that synchronous release requires SNAP-25, the substrate for BoNT/A LC, as well as intact synaptobrevin, the substrate for BoNT/B, /D and /F (Simpson, 2004).

4.3. K^+ channel blockers in human botulism

The K^+ channel blockers guanidine and 3,4-DAP have been evaluated in a small number of human botulism cases. The general findings were that while these blockers produced some increase in muscle strength, their use did not lead to the return of spontaneous ventilation (Cherington and Schultz, 1977; Davis et al., 1992). It is not clear if respiratory muscles are less responsive to K^+ blockers than limb or postural muscles or if the doses used clinically were insufficient to reverse muscle paralysis (Davis et al., 1992). High doses of 3,4-DAP were not attempted in patient studies to avoid the risk of seizures and other potential side effects. Based on the marked differences in 3,4-DAP concentrations that are effective in reversing BoNT/A intoxication in isolated diaphragm muscle ($\geqslant$10 μM; Figs. 2A and 3B), and the plasma levels in patients receiving the maximum tolerated dose of 3,4-DAP for conditions such as amyotrophic lateral sclerosis (1.2 ± 0.5 μM) (Aisen et al., 1995), it is likely that BoNT-intoxicated patients did not receive a dose that was sufficient to reverse paralysis in the case reports of Cherington and Schultz (1977) or Davis et al. (1992).

4.4. Development of more effective K^+ channel blockers

At the present time, the K^+ blockers hold promise as potential therapeutic agents, but additional strategies, such as development

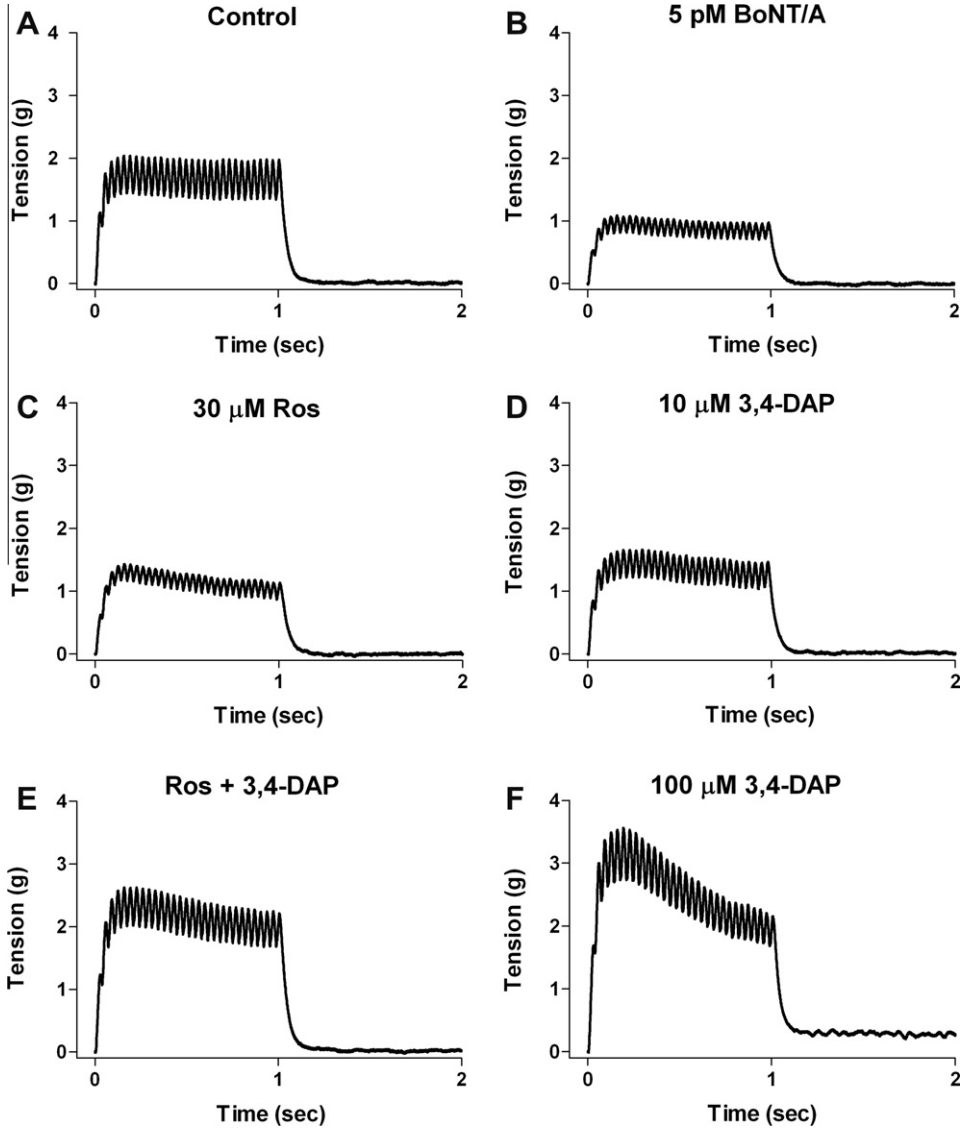

Fig. 5. Effect of 3,4-DAP and Ros on tetanic tensions elicited with 1-s long trains at 30 Hz. Tensions (amplitude and area) were depressed after exposure to 5 pM BoNT/A (B). Tensions were increased slightly by 30 μM Ros (C) but more substantially by 10 μM 3,4-DAP (D). The combination of 30 μM Ros and 10 μM 3,4-DAP raised tensions to values exceeding control (E). Tetanic tensions were enhanced even more markedly by 100 μM DAP; however, this concentration also produced tetanic fade and baseline instability (F).

of more selective compounds, and targeting of the inhibitors to neuromuscular and neuroeffector synapses, will be required to exploit their full potential. With regard to more selective inhibitors, Mayorov et al. (2010) synthesized new analogs of 3,4-DAP with the goal of finding compounds that displayed both an enhanced affinity for nerve terminal K^+ channels and a reduced propensity to cross the blood brain barrier. Although, none of the analogs were more potent than 3,4-DAP, one was found to have a more favorable peripheral to CNS distribution (Mayorov et al., 2010). Complicating the search for aminopyridines with low CNS toxicity is that their binding site on the K^+ channel is accessible only from the cytoplasmic membrane surface (Howe and Ritchie, 1991; Muñoz-Caro and Niño, 2002). This makes the goal of finding compounds with reduced CNS penetration challenging, since such compounds would also have an impaired ability to gain access to the cytoplasmic surface of the membrane. For this reason, it may be profitable to also consider K^+ channel blockers that act on the outer surface of the membrane in future studies.

4.5. Combination of K^+ channel blockers with Ca^{2+} channel activators

To address the issue that only high and potentially toxic doses of 3,4-DAP can antagonize the actions of BoNT, we examined the effect of combining 3,4-DAP with Ros, a trisubstituted purine analog best known for its action as a cyclin-dependent kinase (CDK) inhibitor (Fig. 1) (Benson et al., 2007). Distinct from its action on cell cycle regulation, Ros is also a novel activator of neuronal N-, P/Q- and R-type Ca^{2+} channels, and acts by slowing channel deactivation, thus prolonging channel open state (Buraei et al., 2005, 2007). These channels have been implicated in the regulation of transmitter release (Yan et al., 2002; DeStefino et al., 2010). Ros has been examined for treatment of human immunodeficiency virus type-1 (Guendel et al., 2010) and advanced malignancies (Le Tourneau et al., 2010), and is in Phase II trials for non-small cell lung cancer and nasopharyngeal carcinoma (Aldoss et al., 2009). Importantly, the plasma concentrations of Ros found in clinical trials are in the range of those used in the present study (Benson et al., 2007).

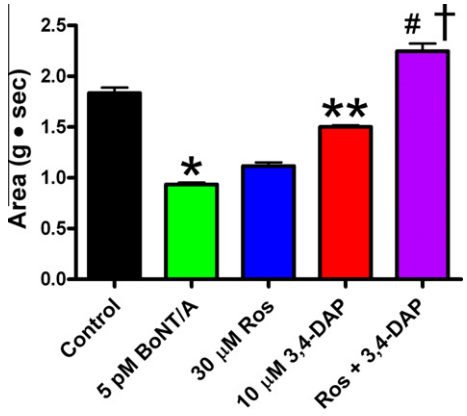

Fig. 6. Histograms showing the effects of 30 μM Ros, 10 μM 3,4-DAP and combination of the two drugs in hemidiaphragm muscles intoxicated with 5 pM BoNT/A. Data shown are mean ± SEM of integrated tension records obtained from 5 to 9 hemidiaphragm muscles per condition. Recording were made 20–30 min after drug addition. Ros and 3,4-DAP were added to separate muscles, and combinations were achieved by subsequent addition of Ros or 3,4-DAP, as appropriate. Differs significantly from control ($^{*}P < 0.001$); differs significantly from values in the presence of BoNT/A ($^{**}P < 0.05$, $^{\#}P < 0.001$); differs significantly from values observed in 10 μM 3,4-DAP ($^{\dagger}P < 0.01$).

Unlike 3,4-DAP, Ros was not able to reverse BoNT-mediated depression of twitch tension on its own. However, when 30 μM Ros and 10 μM 3,4-DAP were co-applied, muscle tensions were restored rapidly to near control levels for twitch tension (Fig. 3) and above control for tetanic tension (Fig. 6). These results are encouraging since they demonstrate the possibility of achieving a prompt recovery from paralysis by using drugs with synergistic mechanisms of action: increased Ca^{2+} influx via K^{+} channel blockade (Molgó et al., 1980) and enhanced Ca^{2+} conductance via prolongation of the channel open time (Yan et al., 2002; Buraei et al., 2005, 2007). Although the concentrations of 3,4-DAP used in this study (10–100 μM) are toxic systemically (Aisen et al., 1995), the concentration of Ros falls within values for serum levels that are well-tolerated by patients (Benson et al., 2007). Based on the success of targeting Ca^{2+} entry by two complementary approaches, it is reasonable to assume that by making even modest improvements in the margin of safety of the K^{+} channel blocker and in the potency of the Ca^{2+} channel activator, we should be able to develop a safe and effective treatment for BoNT/A intoxication.

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